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REVIEW ARTICLE

NF-Y and the transcriptional activation of CCAAT promoters

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Abstract
The CCAAT box promoter element and NF-Y, the transcription factor (TF) that binds to it, were among the first cis-elements and trans-acting factors identified; their interplay is required for transcriptional activation of a sizeable number of eukaryotic genes. NF-Y consists of three evolutionarily conserved subunits: a dimer of NF-YB and NF-YC which closely resembles a histone, and the “innovative” NF-YA. In this review, we will provide an update on the functional and biological features that make NF-Y a fundamental link between chromatin and transcription.

The last 25 years have witnessed a spectacular increase in our knowledge of how genes are regulated: from the identification of cis-acting sequences in promoters and enhancers, and the biochemical characterization of the corresponding TFs, to the merging of chromatin studies with the investigation of enzymatic machines that regulate epigenetic states. Originally identified and studied in yeast and mammals, NF-Y – also termed CBF and CP1 – is composed of three subunits, NF-YA, NF-YB and NF-YC. The complex recognizes the CCAAT pentanucleotide and specific flanking nucleotides with high specificity (Dorn et al., 1997; Hatamochi et al., 1988; Hooft van Huijsduijnen et al. 1987; Kim & Sheffery, 1990). A compelling set of bioinformatics studies clarified that the NF-Y preferred binding site is one of the most frequent promoter elements (Suzuki et al., 2001, 2004; Elkon et al., 2003; Mariño-Ramírez et al., 2004; FitzGerald et al., 2004; Linhart et al., 2005; Zhu et al., 2005; Lee et al., 2007; Abnizova et al., 2007; Grskovic et al., 2007; Halperin et al., 2009; Häkkinen et al., 2011). The same consensus, as determined by mutagenesis and SELEX studies (Bi et al., 1997), was also retrieved in ChIP-on-chip analysis (Testa et al., 2005; Ceribelli et al., 2006; Ceribelli et al., 2008; Reed et al., 2008). Additional structural features of the CCAAT box – position, orientation, presence of multiple Transcriptional Start Sites – were previously reviewed (Dolfini et al., 2009) and will not be considered in detail here.

Keywords: NF-Y, transcription, CCAAT box, post-translational modification, transcription factors and cofactors

The structure of NF-Y

In the second half of the 1990s, it became obvious that two NF-Y subunits are related to core histones, NF-YB to H2B and NF-YC to H2A (Baxevanis et al., 1995). Histones are highly conserved proteins whose dimeric and tetrameric interactions – in the case of H3/H4 – result in an octameric structure, which wraps 146 nucleotides of DNA into a nucleosome, the basic unit of chromatin (Luger et al., 1997). The globular domain of histones – termed Histone-fold Domain, HFD – is directly responsible for the formation of the octamer and the non sequence-specific contacts with DNA. The identification of HFDs in NF-YB and NF-YC by BLAST searches was corroborated as important for dimer formation and DNA-binding by mutagenesis studies of several HFD residues shared with core histones (Xing et al., 1993; Sinha et al., 1996; Kim et al., 1996; Zemzoumi et al., 1999). Residues required for interaction of NF-YA/NF-YB with NF-YA – map to areas either divergent from core histones, such as the NF-YC αC helix, or are scattered on divergent aminoacids of the otherwise conserved α2 helix of NF-YB. At the same time, additional proteins were reported to contain HFDs (Figure 1): the TBP/TATA-binding NC2α/βtranscriptional repressor (Zhou et al., 2009 and References therein); several TAFs, subunits of the TBP-containing TFIID and of the SAGA and ATAC coactivator complexes (Reviewed by Gangloff et al., 2001; Nagy & Tora, 2007). The ISWI-containing nucleosome remodeling Chrac complex of *Drosophila* associates with small subunits containing HFDs (Poot et al., 2000; Corona et al., 2000), also identified

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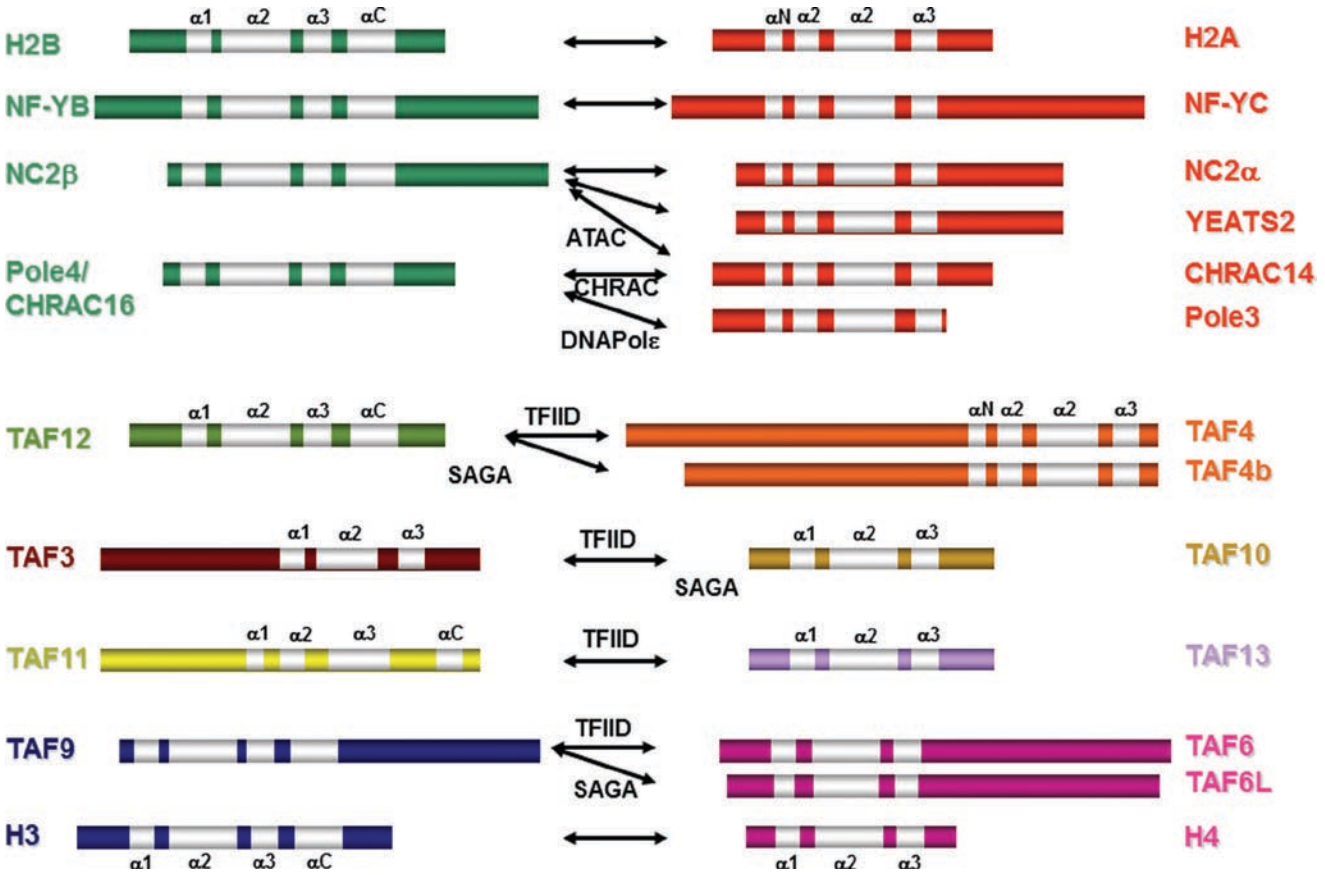

Figure 1. HFD proteins. HFD proteins are associated according to their resemblance to the respective core histones. The complexes in which the dimmers are found are indicated.

by out group based on homology with NF-YC and NF-YB (Bolognese et al., 2000). DNA polymerase ε involved in DNA-repair, has two small subunits, Pole3, the human homologue of Chrac16, and Pole4. Furthermore, biochemical studies recently showed that NC2β in partnership with hChrac14 and a new HFD protein, YEATS2, are components of ATAC complexes (Suganuma et al., 2008; Wang et al., 2008).

Typically, only 15–18% protein identity is observed along the 65–80 aminoacids of HFDs of different subclasses, but NC2α/β and Chrac14/Pole3/Pole4 show far greater resemblance to NF-YB/NF-YC, with 30% identity. This subclass of HFDs seem to be more closely related to H2A/H2B than to H3/H4. Crystallographic studies of NC2α/β heterodimers with TBP and the TATA box definitively documented the presence of a HFD, (Kamada et al., 2001; Romier et al., 2003; Hartlepp et al., 2005). We reported that heterodimers are not formed between recombinant NC2α/β and NF-YB/NF-YC (Zemzoumi et al., 1999), but the promiscuous nature of Pole4 and NC2β dimerization indeed begs the question of whether NF-YB could also have alternative partners: more thorough biochemical analyses *in vivo* are required to exclude this possibility.

The structure of the NF-YA subunit is unknown. Biochemical data indicates that the evolutionarily conserved domain is composed of two helices (Xing et al., 1993, 1994; Mantovani et al., 1994), each specifying a function: A1 mediates NF-YB/NF-YC interaction, and A2 specifies CCAAT-binding through an unknown mechanism. We proposed a model which is based on how histones contact and bend DNA in nucleosomes, and on the mutagenesis, biochemical and genetic experiments on CCAAT elements (Romier et al., 2003): further structural studies will hopefully shed light on how NF-YA can turn an HFD dimer with little sequence-specificity into a highly selective complex.

NF-Y and histone PTMs

Core histones are subject to a plethora of post-translational modifications – PTMs – which play a crucial role in the definition of chromatin territories (Suganuma & Workman, 2011). The peculiar histone-like features of NF-Y and its early involvement to set the stage for transcriptional activation invited studies to define the relationships between NF-Y binding and histone PTMs. Essentially, NF-Y was transiently removed from DNA by two methods: overexpressing a dominant negative NF-YA, or functionally inactivating subunits by RNAi. The results are summarized in Figure 2. There are several points worth noting: (i) histone methylation associated with activation and elongation – H3K4me3, H3K36me3 and H3K79me2 – depend on NF-Y (Donati et al., 2008;

NF-Y-DEPENDENT PTMs	NF-Y-INDEPENDENT PTMs
H2BK120ac ↑	H2AK5ac
H2BK16ac ↓	H2AK9ac
H3K4me1 ↓	H3K4me2
H3K4me3 ↓	H3K9ac
H3K18ac ↑	H3K14ac
H3K27ac ↑	H3K27me3
H3K36ac ↑	H3K56ac
H3K36me3 ↓	H4K5,8,12,16ac
H3K79me2 ↓	H4K91ac
H4K20me3 ↑	

Figure 2. NF-Y and histone PTMs. A list of histone PTMs whose presence, or absence, in promoters are dependent (left) or independent (right) of NF-Y binding.

Gurtner et al., 2008; Gatta & Mantovani, 2008). (ii) Acetylation of H3K9 and K14 and of H4 are, in general, little affected by NF-Y removal (Gatta & Mantovani, 2011). (iii) Other regulatory acetylations on H3K18, H3K27, H3K36 and H2BK120 are increased by NF-Y removal (Gatta & Mantovani, 2011); these residues can be modified by alternative PTMs – methylations and monubiquitination of H2BK120 – suggesting that NF-Y is involved in a switch of modifications. Work on the terminally differentiated myotube cells suggests that heterochromatic marks – H3K9me3 and H4K20me3 – are deposited on silenced cell cycle genes upon NF-YA removal in differentiated cells (Gurtner et al., 2008). The picture is particularly intriguing for methylations of H3K4, which are mediated by the MLL complex (Dou et al., 2006). NF-Y is required for mono – and tri-methylate, but not dimethylate H3K4 (Donati et al., 2008; Gatta & Mantovani, 2008). The Lysine demethylase KDM1 (LSD1), which removes H3K4me2, and coREST, a cofactor important for KDM1 activity (Qureshi et al., 2010), are recruited by NF-Y, thus supporting the di- to mono-methylation switch. Interestingly, coREST is a nucleosome interacting protein (Yang et al., 2006). Additionally, NF-Y apparently affects the local composition of MLL complexes by contacting the hASH2L subunit, specifically required to impart tri-methylation (Fossati et al., 2011). Additional proof of MLL1/NF-Y interplay was obtained from studies on the MDR1 promoter (Huo et al., 2010). Interestingly, genetic and biochemical work indicates that H3K4me3 is downstream of H3K79me2 and of H2BK120 monoubiquitination, the latter being one of the earliest marks of active genes (Suganuma & Workman, 2011). This raises the possibility that a similar modification on related NF-YB Lysine(s) could establish histone methylations in promoter environments. Another key issue is how NF-Y recognizes the CCAAT box in nucleosome-reconstituted chromatin. Studies on the rules of nucleosome positioning in yeast found that CCAAT boxes are among the few TF binding sites left "open" for free access in promoters by nucleosome positioning (Segal et al., 2006); *in vitro* and *in vivo* studies suggest that NF-Y can be recruited by H3/H4 in active promoters, replacing H2A/H2B (Caretti et al., 1999; Gatta & Mantovani 2008). In summary, NF-Y behaves as a promoter organizer, making use of the H2A/H2B-like structure for transient, or stable, H3/H4 interactions which prevent nucleosome formation on core promoters.

The biology of NF-Y

We will summarize the latest advances in different organisms, with the exception of plants, because their complexity and the rapidly expanding biotechnological interest invite a specific review devoted to their numerous NF-Y subunits.

NF-Y in yeast

The yeast *S. cerevisiae* is an aerobic-anaerobic facultative organism. When grown in a medium containing glucose, it produces energy and ethanol through glycolysis and fermentation, while the genes encoding proteins involved in the respiratory metabolism are repressed. When non-fermentable carbon sources, such a lactate or glycerol, are available, yeast cells switch to oxygen-fueled metabolism. The switch is similar to conditions in which glucose is exhausted and the end product, ethanol, is used as a carbon source, together with respiration: in this diauxic shift respiratory genes are induced (DeRisi et al., 1997). In the 1980s, screenings for genes essential to grow on non-fermentable carbon sources allowed the lab of L. Guarente to isolate HAP2 (NF-YA), HAP3 (NF-YB), HAP4 and, finally, HAP5-NF-YC (Pinkham et al., 1987; Hahn et al., 1988; Forsburg et al., 1989; McNabb et al., 1995; McNabb & Pinto, 2005). The HAP complex binds to the CCAAT sequence in the UAS (*U*pstream *A*ctivation *S*equence) of numerous cytochrome genes, and it is a master regulator of respiratory metabolism (Buschlen et al., 2003; Schüller, 2003), interchangeable with the mammalian complex (Chodosh et al., 1988). Analysis of expression profiles after deletion of HAP2 or HAP3, and in cells undergoing a diauxic shift, identified genes whose expression changed: the CCAAT box is clearly enriched in their UAS (Bonander et al., 2008, and References therein). Strong evidence indicates that almost all nuclear encoded respiratory genes contain a CCAAT box in their promoters and are activated by the HAP2/3/4/5 complex (Buschlen et al., 2003).

The HAP2/3/5 subcomplex was shown to be constitutively expressed but devoid of activating activity, a function provided by HAP4, the subunit induced after the glucose-to-lactate shift, through a typical strong acidic activation domain (Forsburg et al., 1989). Evolution has taken an interesting twist, since HAP4 is only present in *fungi* (Bourgarel et al., 1999; Kato, 2005; Mercier et al., 2006), while in other higher eukaryotes, the activation function of HAP4 has been structurally modified, split and incorporated into NF-YA and NF-YC, under the forms of two large Glutamine- and hydrophobics-rich domains (Li et al., 1992; Coustry et al., 1996; di Silvio et al., 1999).

NF-Y in *Drosophila*

dmNF-YA overexpression and knockdown experiments showed lethality at various developmental stages (Yoshioka et al., 2007). As for the pathways implicated, genetic experiments suggest a cooperation with the *eyeless* gene and a negative interplay with *Distal-less*. In a second report, KO of dmNF-YA in the notum compartment of wing discs resulted in a thorax disclosed phenotype (Yoshioka et al., 2008). Interestingly, reduction of the *Drosophila* JNK *basket* (bsk) gene enhanced the dmNF-YA-induced KO phenotype and overexpression of Bsk suppressed it; expression from a JNK-dependent reporter was reduced by dmNF-YA RNAi. The bsk promoter contains a CCAAT motif bound by dmNF-Y *in vivo*. Finally, the interplay is not limited to wing disks, as bsk mRNA is reduced in dmNF-YA KO *larvae*. These results clearly establish an important genetic link between NF-Y and the JNK pathway that could well be conserved beyond insects.

dmNF-YC was recovered in two unrelated genetic screens. The first was a microarray profiling experiment aimed at identifying target genes of *Dorsal*, a TF important for early embryogenesis: one was dmNF-YC, expressed in a tissue-specific manner, which turned out to be essential for the activation of mesodermal genes (Stathopoulos et al., 2002). The second was a screen for genes able to guide specifically R7 and R8 photoreceptor axons to different synaptic layers in the brain, a process controlled positively and negatively by TFs. Namely, the R8-specific TF *Sens* controls R8 targeting by binding to and activating the gene of the R8-specific cell-surface protein *Capricious*. Instead, *Prospero* positively guides R7 targeting, together with active repression of R8 by dmNF-YC: genetic ablation of dmNF-YC leads R7 axons to terminate in the same layer as R8 axons; importantly, genetic experiments indicate that this is due to the derepression of the *Sens* gene late in R7 differentiation (Morey et al., 2008).

NF-Y in C. elegans

The *C. elegans* egl-5 – ortholog of *Drosophila* Abdominal-B – is a Hox TF expressed in the tail and involved in pattern formation. Mutations of the ceNF-YA-1, but not of the related ceNF-YA-2, nor ceNF-YB-1 or ceNF-YC-1 genes, result in several developmental defects, and the ectopic expression of egl-5. The egl-5 promoter has a canonical CCAAT box, whose mutation leads to inappropriate derepression of the gene. Moreover, NF-Y interacts with the MES-2/MES-6 subunits of the repressive PcG complex (Deng et al., 2007). This study established a physiological role of NF-Y in restricting expression patterns of egl-5, and a first link to Polycomb repression: coupled with the data from *Drosophila* mentioned above, they represent a strong genetic proof of the repressive role of NF-Y.

NF-Y in *Schmidtea mediterranea*

The NF-YB homologue of this planarian was identified in a genetic screen of genes required for male germ cell development. Inactivation of this subunit resulted in a phenotype consistent with an inability to maintain sperm stem cells, while late stages of spermatocyte differentiation remained unaffected (Wang et al., 2010). Incidentally, NF-YB mRNA is highly abundant in mouse spermatogonia with respect to other cell types (Chalmel et al., 2007), supporting the idea that it is indeed involved in testis stem cell biology beyond planarians.

NF-Y in zebrafish

Surprising findings were reported in a study focusing on NF-YB in *D. rerio*. Expression is maternally inherited, which is expected, but it is then restricted to head cartilages and the developing notochord. Injection of a drNF-YB morpholino led to smaller head, sharpen Meckel's cartilage, loss of ceratobranchial cartilage, and enlarged angles of ceratohyal cartilage. TUNEL assays and staining with neural crest cells markers indicate that they undergo apoptosis, affecting cartilage formation and causing the head phenotypes (Chen et al., 2009). Intriguingly, an extremely similar phenotype is observed by injecting "unspecific" morpholinos known to activate the p53 pathway, also leading to apoptosis in the same compartments (Robu et al., 2007). The authors did rescue the phenotype by injecting capped drNF-YB mRNA, hence proving it to be a specific effect. However, as siRNA interference of human NF-YB also triggers p53-dependent apoptosis (see below), it is possible that part of the drNF-YB KO phenotype is triggered *via* drTP53 activation.

NF-Y in mammals

Studying NF-Y by genetic experiments in mammals extremely challenging. KO technology on CBF-B (NF-YA) in mice resulted in death very early in embryogenesis, with cells affected by severe defects in S-phase progression and massive apoptosis (Bhattacharya et al., 2003). Functional inactivation of the subunits by shRNA interference are more manageable, but only in transient experiments, resulting in (i) a delay in G2/M exit upon targeting of NF-YB or NF-YC, and (ii) a delay in S-phase progression, followed by apoptosis caused by p53 activation, upon NF-YA elimination (Benatti et al., 2008, 2011). The reason for the different phenotypes is unclear, but expression profile data suggest that NF-YB/NF-YC and NF-YA, while sharing CCAAT recognition, might have separate functions. It was reported that certain post-mitotic cells, such as differentiated myotubes and circulating monocytes, are devoid of NF-YA (Farina et al., 1999; Marziali et al., 1999; Gurtner et al., 2003; Bevilacqua et al., 2002), but not of NF-YB, and presumably of NF-YC, as if the HFD subunits were part of NF-YA-independent regulatory circuits, whose future identification is clearly a priority.

Regulation of the subunits

NF-Y splicings

It is now widely accepted that the vast majority of mammalian genes are differentially spliced, and that this compensates for the relative "paucity" of the overall number of genes, when compared to genomes of simpler

organisms. NF-Y is no exception to the rule (Figure 3). Specifically, it has long been known that NF-YA has two major isoforms (Li et al., 1992). The difference is only 28 additional aminoacids in the Q-rich activation domain, with no notable features: how this affects activation of specific sets of genes is an open question. In at least one example – the Cystathionine synthase promoter – cooperation with Sp1, another Gln-rich TF, was shown to be dependent upon the specific isoform coexpressed (Ge et al., 2001). HOXB4 transcription in stem cells is governed by NF-Y sites and an E-box in the promoter and an intronic enhancer including a YY1 responsive element (Gilthorpe et al., 2002; Zhu et al., 2003, 2005). HOXB4 is a master gene for Hematopoietic Stem Cells (HSC) and overexpression of the NF-YA short isoform – NF-YAs – led to an increase in expression of several HOX genes, as well as in TFs mediating Notch and Wnt signaling, notoriously crucial in HSC maintenance. Retroviral infections of NF-YAs in mouse HSCs increased substantially their capacity to repopulate the bone marrow of immunocompromized animals (Zhu et al., 2005). Strikingly, this experiment was recently repeated using NF-YAs fused to a TAT peptide, providing a CPP (Cell Penetrating Peptide): recombinant NF-YAs added to the medium efficiently enters HSCs *in vivo*, promoting an expansion of population; in turn, this results in better engraftment of bone marrow transplantations (Domashenko et al., 2010). In addition, expression profiling of human and mouse embryonic stem (ES) cells led to the identification enrichment of NF-Y sites in active enhancers (Grskovic et al., 2007). The authors showed by RNAi that NF-YA and NF-YB are required for ES viability; more interestingly, they noticed a switch of NF-YA isoforms upon differentiation toward neuroectodermal lineages: NF-YAs, which predominates in ES cells, is replaced by NF-YAl. Therefore, there are now several indications that NF-Y plays important roles in stem cells, and that NF-YAs could be viewed as the "stemness" isoform.

A first indication that NF-YC is differentially spliced came from a 2-hybrids screening of SMADs interactors, which identified an NF-YC cDNA lacking exon 4, and hence a large parte of the HFD (Chen et al., 2002). We then detailed several NF-YC isoforms, in part resulting from the presence of two promoters, in part to differential splicings of exons 8 and 9 (CeribelLi et al., 2009). The larger insertion – 82 aminoacids – lacks a Q-rich domain, being rich in charged residues. It will also be interesting to study NF-YC splicing isoforms in stem cells and verify whether they have specific NF-YA/NF-YC combinations.

NF-YB differential splicing has not been observed in mammals. However, two non-canonical isoforms were found in toad oocytes: one contains an insertion in the 5′UTR, the second contains 32 additional aminoacids in the L1 of the HFD (Yan & Tso, 2004). The "canonical"

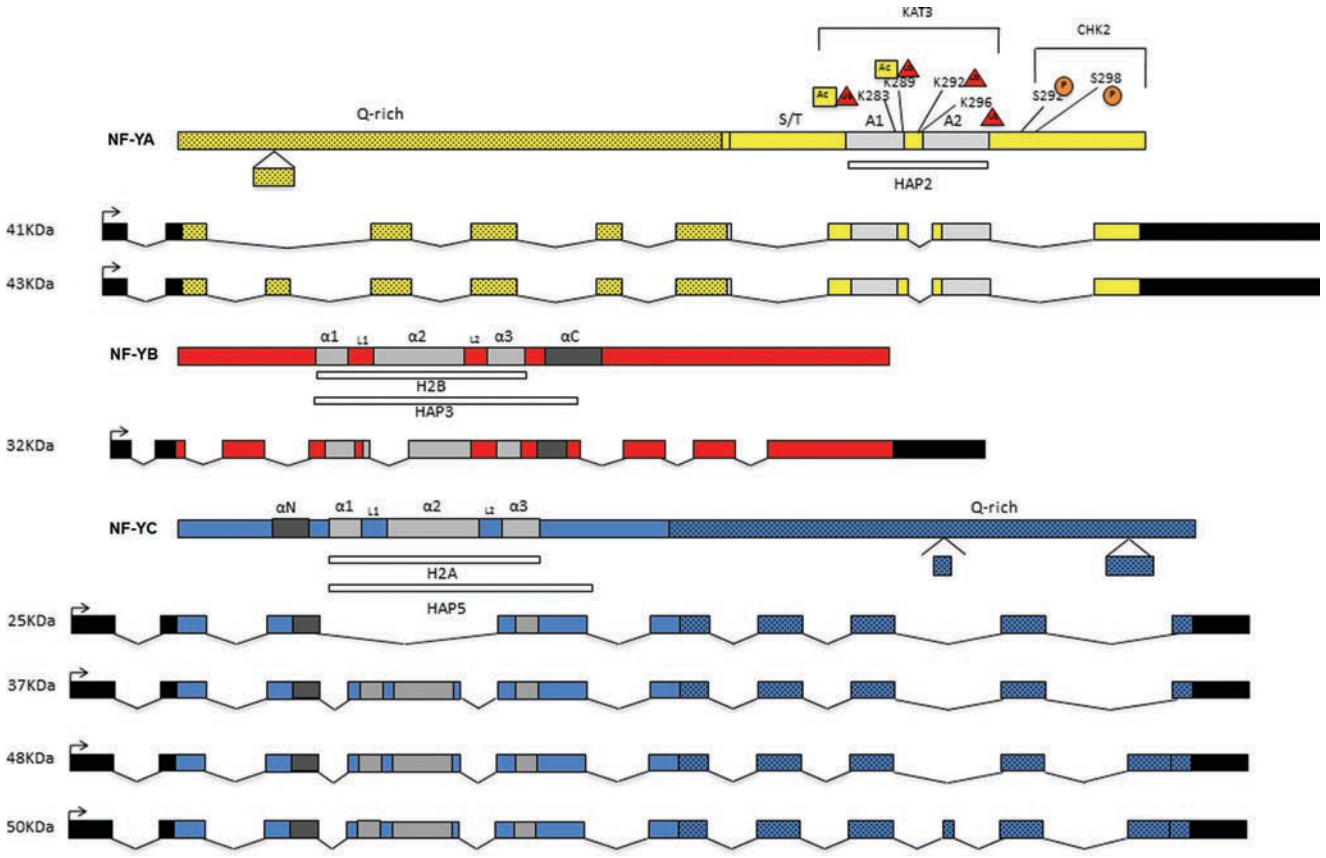

Figure 3. The structure of NF-Y genes. A scheme of NF-YA, NF-YB and NF-YC genes is depicted, including functional domains, differential splicings and residues involved in post-translational modifications.

NF-YB was present in low temperature oocytes (LTE), which are bound to mature correctly, and absent in high temperature oocytes (HTE), which fail to do so. The long isoform, instead, was abundant in HTE: it might well be unable to bind to DNA, as the additional aminoacids interrupt a region involved in important DNA-binding contacts, so it could represent a natural dominant negative isoform of NF-YB.

Expression of the subunits

To date, neither immortalized nor transformed cell lines have been described in which any of the three subunits are not expressed. This is not surprising, considering the importance of NF-Y for activation of genes promoting cellular growth. However, certain types of normal post-mitotic cells are without NF-YA: in myocytes, the protein level drops during terminal differentiation to myotubes, it is absent in circulating monocytes and it increases when cells are activated to become antigen-presenting macrophages (Farina et al., 1999; Marziali et al., 1999; Gurtner et al., 2003). NF-YA levels also progressively decrease in primary cells undergoing senescence (Matuoka & Chen, 2002). Several reports describe the increase of NF-Y proteins following different treatments: MMS increases NF-YA, leading to activation of the OGG1 and GADD45 promoters (Lee et al., 2004; Zumbrun et al., 2009). Increased NF-Y activity is observed following TGFβ (Lindahl et al., 2002; Eggen et al., 2001), AKT (Lee et al., 2005), MERK1/2 and ERK1/2 activation (Yokota et al., 2010). By way of contrast, a decrease in activity was reported after addition of Sinvastatin, A23187, Okadaic acid, TNFα, anthraciclin (Louneva et al., 2006; Zhu et al., 2007a, 2007b; Finch et al., 2001; Morin et al., 1995; Park et al., 2002; Park et al., 2011). It is formally possible that these changes are due to altered transcriptional activity, but we suspect that NF-Y PTMs might rather be involved.

Post-translational modifications

Currently, two types of PTM – acetylation and phosphorylation – are reported for NF-Y subunits. NF-YB was shown to be acetylated by KAT3B (p300), KAT2A (hGCN5), and NF-YA by KAT3B (Li et al., 1998; Currie, 1998; Manni et al., 2008). Indeed, studies reporting protein-protein interactions with these KATs are numerous (Figure 3). KAT3B acetylates the six Lysines in the conserved domain, located in the A1 helix and in the linker region of NF-YA. These modifications prolong the – relatively – short half-life of the subunit by a classic mechanism: preventing poly-ubiquitination-mediated protein degradation by the proteasome (Manni et al., 2008). However, it is unknown whether these PTMs affect interactions between subunits, DNA-binding or transcriptional activation. Equally unclear is the role of NF-YB acetylation, which is apparently important for transcriptional induction (Li et al., 1998), and nothing is known about the residues involved. This topic is of interest, as recent studies with HDAC inhibitors suggest that part of their anti-proliferative activities might be due to hyperacetylation of proteins unrelated to core histones, possibly including NF-Y. Specifically, NF-YA acetylation increases in confluent rat PC6 cells, leading to increased DNA-binding and expression of RGS4 (Yang et al., 2010).

As for phosphorylation, two serines at the C-terminal of helix A2 of NF-YA – S292 and S298 – are targeted by CDK2 (Yun et al., 2003; Chae et al., 2004; Chan et al., 2010). A mutant harboring acidic residues behaves as a dominant negative in cell cycle progression, blocking cells at the G1/S boundary. This result highlights the role of NF-Y in cell cycle regulation, inserting NF-YA among the key CDK2 targets: because drugs inhibiting CDK2 have shown anti-proliferative potential (reviewed by Węsierska-Gądek et al., 2011), NF-Y targeting might thus be relevant to understanding one of the mechanisms of their activity.

Cellular redox

The regulation of the oxidative status is an important parameter in cellular physiology. Specifically, oxidation of Cysteines is known to impact on their tertiary and quaternary structures. NF-Y has long been known to be regulated by redox mechanisms (Nakshatri et al., 1996), and a recent elegant study performed on the *Aspergillus nidulans* homologue AnHapC/E/B clarified the molecular mechanisms leading to alteration under oxidative conditions (Thön et al., 2010). The regulated subunit is AnHapC, which has three conserved Cysteines – equivalent to human NF-YB C83, C87 and C103 – in the α2 helix of the HFD: two of these residues, in particular, sense the cellular redox potential, allowing heterodimerization under reduced conditions. When oxidized, NF-YB forms homodimers, which are located in the cytoplasm: as a result, formation of the trimer, CCAAT-binding and transcriptional activation in the nucleus is impaired. Among several targets, the promoter of the NapA TF gene coordinates the general response to oxidative stress: under reduced conditions, transcription is repressed by AnHapC/E/B, and indeed HapC deletion leads to derepression of the transcriptional program. These experiments provide still more evidence of the physiologic importance of NF-Y-mediated repression.

Nuclear localization

Several studies have focused on the mechanisms underlying the localization of the subunits. The initial report by our group that NF-YA has intrinsic capacity to enter *nuclei*, whereas the HFD dimer enters coordinately, with NF-YB helping NF-YC (Frontini et al., 2004), was confirmed and developed through studies in *A. nidulans* and mammals (Kahle et al., 2005; Steidl et al., 2004; Tüncher et al., 2005; Goda et al., 2005). The nuclear import of NF-YA is determined by interactions between basic residues in the A1 and A2 helices and Importin β, and NF-YB/NF-YC *via* HFD interactions with Importin 13. In particular, Importin 13 competes for NF-YA interactions with NF-YB/NF-YC, confirming that they are imported separately, and that the trimer most likely assembles in

the nucleus (Kahle et al., 2005). Interestingly, the same system is used to import other H2A/H2B-like HFD proteins such as Chrac14/16 and NC2α/β (Kahle et al., 2009; Walker et al., 2009). Regulation of NF-YA nuclear import is apparently influenced by TGFβ, at least in NIH3T3 cells (Alabert et al., 2006); interestingly, SMADs, – the TF effectors of TGFβ signaling and NF-Y interactors – are also relocated in the nucleus following the stimulus (Figure 4). One is left to wonder whether the two TFs travel together ending up on overlapping sets of target promoters. Until now, examples of NF-Y/SMAD coregulation are scant in the literature. Finally, NF-YA nuclear localization is also regulated by TSC-2 (Tuberin), a kidney tumor suppressor which controls the DNA repair enzyme OGG1 (Habib et al., 2008). Haploinsufficiency of Tuberin in humans increases oxidative DNA-damage by decreasing the levels of NF-YA, which controls OGG1 transcription (Lee et al., 2004; Habib et al., 2008). Interestingly, NF-YA is mostly relocated in the cytoplasm of renal tumor cells with a defect in Tuberin expression (Habib, 2009).

NF-Y and fellow TFs

NF-Y was originally characterized as the *N*uclear *F*actor binding to the *Y* box of the promoters of MHC Class II genes (Dorn et al., 1987), acting cooperatively with RFX which binding to the neighboring X box – to constitute a functional unit in promoters and enhancers. The two TFs bind synergistically *in vitro* and *in vivo* (Villard et al., 2000; Zhu et al., 2000; Caretti et al., 2000), presenting a platform to recruit the master MHC Class II coactivator CIITA (Leimgruber et al., 2009, and References therein). A second well-known paradigm is represented by the NF-Y/SREBP combination, which regulates essentially all genes involved in the cholesterol and fatty acid metabolism (Ericsson et al., 1996; Sato et al., 2000; Moon et al., 2000; Kim et al., 2001; Nagai et al., 2002; Schweizer et al., 2002), including the promoters of the SREBP-1 and SREBP-2 TF regulators (Sato et al., 1996; Amemiya-Kudo et al., 2000; Cagen et al., 2005). A ChIP on chip study definitely confirmed that the targets of NF-Y and SREBP-1 overlap significantly (Reed et al., 2008).

Over the last few years, a combination of NF-Y transcriptome, location and bioinformatics analyses has widened the list of NF-Y partners mediating synergistic activation of specific classes of promoters. Among the numerous examples available in the literature, the elements and partner TFs that, in our opinion deserve more immediate attention include the following:

NF-Y protein- protein interactions

	TFs	Fx	Ref.	GTFs	Fx	Ref.	Cofactor	Fx	Ref.	OTHERS	Fx	Ref.
NF-YA	c-JUN	A	Faniello et al, 2002	TAX_1	A/R	Plise-Masison et al, 1997	KAT2B	A/R	Park et al, 2002	p32	R	Chattopadhyay et al, 2004
	SRF	A	Yamada et al, 1999b	TAF4	A/R	Frontini et al, 2002	ASH2L	A	Fossati et al, 2011	PML-RAR_α	R	van Wageningen et al, 2008
	C/EBPα	A	Zhu et al, 2004				PC4	A/R	Currie, 1998	E5	A	Grindlay et al, 2005
	Sp1	A	Liang et al, 2001				RNF4		Wu et al, 2004	ACTN4	A	Poch et al, 2004
			Roder et al, 1999									
	USF1	?	Ito et al, 2010							SF-1	?	Ravasi et al, 2010
	ZHX1	?	Wienk et al, 2009							BRCA1	?	Ravasi et al, 2010
			Hirano et al, 2009									Fan et al, 2002
			Yamada et al, 1999a-1999b									
	ZHX2	R	Wienk et al, 2009							HMGA1	?	Ravasi et al, 2010
			Kawata et al, 2003									
	ZHX3	?	Ravasi et al, 2010							SFRS1	?	Ravasi et al, 2010
	HNF-4	A	Ueda et al, 1998							PWP1	?	Ravasi et al, 2010
	OCT1	A	Ravasi et al, 2010							PAPOLG	?	Ravasi et al, 2010
										CDK2	R	Yun et al, 2003
NF-YB	p73	R	Ravasi et al, 2010	TBP	A	Fang et al, 2004	CIITA	A	Zhu et al, 2000	$NC2_\alpha$	?	Ravasi et al, 2010
						Bellorini et al, 1997			Zika et al, 2003			
	C/EBPα	A	Ravasi et al, 2010	TAF11	R	Frontini et al, 2002	KAT3B	A	Li et al, 1998	YBX1	?	Ravasi et al, 2010
									Faniello et al, 1999			
	C/EBPζ	A	Ravasi et al, 2010	TAF13	R	Frontini et al, 2002	KAT2A	A/R	Currie, 1998	CSDA	?	Ravasi et al, 2010
	ELF1	?	Ravasi et al, 2010	TAF12	A	Frontini et al, 2002	KAT2B	A/R	Peng et al, 2007	RFX	A	Caretti et al, 2000
	Sp1	A	Ravasi et al, 2010	TAF4	A/R	Frontini et al, 2002	ASH2L	A	Fossati et al, 2011			
	MYC	R	Hackzell et al, 2002	TAX_1	A/R	Plise-Masison et al, 1997	coREST	A	Gatta et al, 2008			
			Ravasi et al, 2010									
							HSP-CBF	A	Imbriano et al, 2001			
							RNF4		Wu et al, 2004			
NF-YC	p53	R/A	Imbriano et al, 2005	TBP	A	Fang et al, 2004	CIITA	A	Zhu et al, 2000	RFP	R	Ravasi et al, 2010
						Bellorini et al, 1997						
	p63	R	Testoni et al, 2006	TAF11	R	Frontini et al, 2002	HDAC1	R	Kato et al, 2009	FNLA	A	Bandyopadhyay et al, 2010
	SMAD2	A	Chen et al, 2002	TAF13	R	Frontini et al, 2002	ASH2L	A	Fossati et al, 2011			
	SMAD3	A	Chen et al, 2002	TAF12	A	Frontini et al, 2002	RNF4		Wu et al, 2004			
	USF1,2	?	Ito et al, 2010	TAF4	A/R	Frontini et al, 2002						
			Zhu et al, 2005									
	ATF6B	A	Ravasi et al, 2010	TAX_1	A/R	Plise-Masison et al, 1997						
	MYC	R	Ravasi et al, 2010									
	ATF6	?	Ravasi et al, 2010									
	MR	R	Mural- Takeda et al, 2010									

Figure 4. NF-Y interactors. A comprehensive catalogue of NF-Y interactors, divided according to the subunits and to the overall classification in TFs, General Transcription Factors (GTFs), cofactors and other proteins of unclear transcriptional function. The functional outcome of the interaction is divided in A, activation, and R, repression.

(i) *GC boxes* are extremely abundant promoter elements, rivalling CCAAT in terms of overall distribution near TSSs (FitzGerald et al., 2004). A connection between NF-Y and Sp1 has emerged frequently in the literature (see Dolfini et al., 2009), and their synergistic relationship has been studied in co-expression experiments (Yamada et al., 2000; Ge et al., 2001, 2002). ChIP on chip experiments confirmed a substantial overlap in promoters (Reed et al., 2008). Bioinformatics analysis identified a strong positional bias of GC boxes, with two peaks at 15 and 27 nucleotides downstream of CCAAT, suggesting that NF-Y sites often have nearby GC boxes. Examples of dual functionality with such distance are presented in Supplementary Table 7 of Dolfini et al., 2009. These results are not simple to interpret as GC boxes are recognized by a very large family – >15 members – of zinc-finger TFs with nearly indistinguishable binding specificities including Sp1 and KLFs (Kaczynski et al., 2003). Dissecting individual partnerships will be a demanding exercise.

(ii) *E2Fs* are a family of TFs involved in the regulation of cell-cycle and growth related genes (Blais & Dynlacht, 2004; Attwooll et al., 2004). DNA-binding of prototypical members has been studied in detail and an *in vitro* consensus derived (Kel et al., 2001). *In vivo*, only a minority of sites bound by E2Fs harbor the consensus (Bieda et al., 2006), leading to the suggestion that E2Fs bind predominantly through other TFs, without contacting DNA directly. This view has been revised through ChIP-Seq experiments with E2F1 mutants proving that the DNA-binding domain is indeed required (Cao et al., 2011). There is a genetic link between NF-YA and E2F1, since apoptosis mediated by overexpression of NF-YA is abolished in E2F1$^{-/-}$ mouse fibroblasts (Gurtner et al., 2010). NF-Y indeed activates E2F1 transcription (van Ginkel et al., 1997; Wang et al., 1999), suggesting that NF-Y-mediated E2F1 overexpression causes apoptosis. This observation might also be relevant to a number of bioinformatic and genomic analyses that apparently link NF-Y to E2F and to members of the p53 family, also important regulators of apoptosis (see below). E2F sites are highly enriched in CCAAT containing cell cycle promoters (Elkon et al., 2003; Linhart et al., 2005), analyzed *in vivo* by ChIP (Caretti et al., 2003). However, the link extends beyond this category (Dolfini et al., 2009). Expression profiling of tumors also identifies the NF-Y and E2F partnership (Figure 5). In general, a specific distance between CCAAT and E2F sites could not be found, but we suspect that analysis of ChIP-Seq data will be required to exclude out a precise mutual interplay although we note that evidence of direct protein-protein interactions is so far lacking. The genes active in the G2/M phase of the cell-cycle are particularly interesting as: (i) they tend to be enriched in "cancer gene" signatures (Tabach et al., 2005); (ii) they are enriched in CDE-CHR, key genetic elements that determine phase-specific regulation (Lucibello et al., 1995; reviewed by Muller & Engeland, 2010); CDE-CHR have distance constraints with CCAAT (Lucibello et al., 1995; Linhart et al., 2005) and ChIP showed the presence of E2F4 in the area. Intriguingly, other TFs shown to bind in this region, in some cases in a timely fashion, are KLF4 (Yoon & Yang, 2004) and B-MYB (Mannefeld et al., 2009), the latter possibly as part of the LINC/DREAM complex, which includes E2F4, and the Rb-related p107 and p130 (Schmit et al., 2007): whether this is the elusive CDE-CHR regulatory complex is a possibility that remains to be biochemically demonstrated.

(iii) *Stress TFs.* Cells are subject to stress *stimuli* to which they respond by activating specific TFs responsible for the necessary, and often radical adjustments in gene expression patterns. NF-Y appears to be instrumental in this response at two levels. The first is that many of the genes of master stress TFs are under direct NF-Y control: p53 family members for DNA-damage (Romano et al., 2006), XBP1 and CHOP/DDIT3 for ER-stress response (Ma et al., 2002; Liu et al., 2009; Huang et al., 2010a, 2010b), HSF1 for heat shock and HIF1α for hypoxia (RM, unpublished). The second level is the synergistic activation of coregulated gene sets: essentially all ER-stress (Yoshida et al., 2000, 2001; Yamamoto et al., 2004; Kabe et al., 2005; Donati et al., 2006; Luo et al., 2008) and HSP promoters (Landsberger & Wolffe, 1995; Li et al., 1998; Imbriano et al., 2001), as well as most DNA-damage gene promoters (Ceribelli et al., 2006) are under NF-Y control. NF-Y is generally bound before induction, allowing newly activated TF(s) to access neighboring binding site, thus "pre-setting" the chromatin stage, according to the definition of A. Wolffe (Li et al., 1998).

Interactions with TFs, coactivators and GTFs: not only activation

Transcriptional activation is the result of a multitude of protein-protein interactions between TFs, cofactors, general transcription factors – GTFs – and complexes that modify and remodel chromatin. Inevitably, the list of proteins deemed to interact with NF-Y is long and rapidly increasing, and its numbering is rapidly approaching the catalogue of Mozart's *Don Giovanni* protégé (Figure 4). We will not comment on single interactions, but rather on the trend that emerges. Different TFs are present, often with multiple family members represented: p53/p63/p73, C/EBPα/ζ, SMAD2/3, E box proteins – USF1/2, MYC – ATF6, interacting with either NF-YA or the HFD dimer. In most cases, the interactions lead to synergistic activation of transcription, typically through neighboring binding sites. Coherent with this observation, coactivators and GTFs (TBP, TAFs) are also contacted, and in some cases, the protein domain(s) responsible have been pinpointed. Overall, these data underline the wide range of connections that a TF regulating some 30% of promoters in eukaryotic genomes is expected to undego. However, our knowledge of the mechanisms and molecular details remain limited and a great deal of *in vitro* and *in vivo* work on single promoter systems is still required.

Notably, the emerging theme is repression by NF-Y, for which there are now solid genetic data in *C. elegans*,

Analysis of TFBS in promoters of genes overexpressed in human cancers.

CANCER TYPE	ENRICHED TFBS	REFERENCE
Human embryonic lung fibroblasts (WI-38) and *in vitro* cancerous transformation process	**NF-Y, E2F,** CDE, ELK1, CHR, CHR-**NF-Y**-CDE	Tabach et al., 2005
Small Cell lung Cancer, Leukemia, Lymphoma DLBCL Squamous Cell Lung Carcinoma Lung adenocarcinoma Breast Cancer, Prostate Cancer Hepatocellular Carcinoma Adrenocorticoal Carcinoma	**NF-Y**	Rhodes et al., 2005
Brest Cancer Estrogen treatment	**NF-Y, E2F**, NRF1	Scafoglio et al., 2005
Anaplastic Thyroid Carcinoma	**NF-Y, E2F**	Salvatore et al., 2007
U87, A549, LAN1, SHEP, NCIH929 Ras inhibitor S-farnesylthiosalicylic treatment	**NF-Y, E2F,** FOS	Blum et al., 2007
Brest Cancer cell metastasis	**NF-Y,** YY1**, E2F**	Thomassen et al., 2008
Brest Cancer Cell Malignant progression	**NF-Y**, ELK1, E2F, NRF1	Niida et al., 2008
Hormone Refractory Prostate Cancer	**NF-Y**, SP1, **E2F1**, CREB1, TFAP2A	Calvo et al., 2009
Burkitt's Lymphoma	**NF-Y, E2F,** ELK4, NF-AT, MYB, Let7, SP1	Goodarzi et al., 2009
Prostate Cancer Fetal Prostate stem cell	**NF-Y, E2F,** ETF/TEAD2, AP2, AhR/Arnt	Blum et al., 2009
HCT116 (colorectal cancer) P27 target genes P27 and P16 targets	**NF-Y, E2F**	Yamanaka et al., 2009
Normal Hematopoietic Stem Cell (HSC)	**NF-Y**, Evi-1, ATF4, IRF1, NF1, Ik1, CMYC/MAX, MAZ	Fosberg et al., 2010
Colorectal Cancer MER/ERK pathway regulated genes	**NF-Y, E2F**	Jurchott et al., 2010
Follicular Lymphoma Breast Cancer HCV infected liver cancer Leukemia Diffuse large B-cell lymphoma (DLBCL) CNS tumor Liver/Lung Cancer GC-DLBCL B lymphoma	**NF-Y** **NF-Y, E2F**	Sinha et al., 2010

Figure 5. Analysis of TFBS in gene expression profilings of tumors. The studies in which the NF-Y site was reported to be enriched are listed, together with the additional TFs with a significant statistical abundance.

Drosophila and *A. nidulans*, with links to Polycomb repressive complexes (Deng et al., 2007; Yoshioka et al., 2008; Morey et al., 2008), and genome-wide evidence, with a substantial number of *loci* associated to negative histone marks, H3K27me3 and H4K20me3 (Ceribelli et al., 2008). Evidence that CCAAT and NF-Y are involved in repression was obtained before, in different systems (Boucher et al., 1995; Peng & Jahroudi, 2002, 2003; Gowri et al., 2003; Hewetson & Chilton, 2003; Bernadt et al., 2005). The mechanisms were better defined in growth regulating genes, such as G2/M promoters or PDGFβ-R, by direct interactions with MYC and p53 family members, which repress transcription *via* NF-Y/CCAAT (Jung et al., 2001; Izumi et al., 2001; Hackzell et al., 2002; Uramoto et al., 2004; Imbriano et al., 2005; Testoni & Mantovani, 2006; Montano, 2009; Goetz et al., 2011). Interactions with the zinc finger ZHX2 and with TAF11/TAF13 are also repressive (Kawata et al., 2003; Frontini et al., 2002). Corepressors, such as HDAC1 are frequently associated – mostly with the HFD subunits (Peng & Jahroudi, 2003; Peng et al., 2007; Kato et al., 2009; Nassiri et al., 2010). The associations with KAT2A/B (GCN5/PCAF) are particularly intriguing, since they are observed in conditions of active transcription, as well as on repressed promoters (Huang et al., 2005; Gatta et al., 2010, 2011). On slightly different note, NF-Y subunits are recruited by several hormone receptors on their binding sites in CCAAT-less promoters including: estrogen receptor (NF-YA), mineralcorticoid receptor (NF-YC) and vitamin D receptor (Farsetti et al., 2001; Koszewski et al., 2004; Jääskeläinen et al., 2005; Murai-Takeda et al., 2010), all with a repressive function. Finally, additional interactions were detected by

systematic yeast two-hybrids screenings of hundreds of TFs, and other assays lacking *a priori* knowledge (Ravasi et al., 2010). Their significance is completely unknown, but certainly worth more specific studies in the future.

NF-Y and disease

Mutations or gross abnormalities of NF-Y subunits have not been described, but a number of reports have pointed to alterations of NF-Y function – expression or DNA-binding – as part of the pathogenetic mechanisms of several diseases of disparate origin. We summarize the most notable examples.

Cancer

Genomic analysis has clarified that CCAAT boxes are abundant in growth-promoting genes and functional inactivation of the subunits and KO experiments proved that the presence of NF-Y is required for cells to grow. NF-Y subunits have not been found to be altered, mutated or grossly overexpressed in cancer, yet, a growing number of profiling experiments support the notion that activation of CCAAT-dependent genes is crucial in changing the transcriptome profiles during transformation. The first hint came from a cellular model of fibroblasts transformed by oncogenic hits and inactivation of tumor suppressors, which identified a set of transforming genes driven by NF-Y and E2Fs in their promoters (Tabach et al., 2005). Data on gene expression profiles of breast (Scafoglio et al., 2006; Niida et al., 2008), colon (Jürchott et al., 2010), thyroid (Salvatore et al., 2007) and prostate carcinomas (Blum et al., 2009; Calvo et al., 2010), as well as leukemias (Forsberg et al., 2010), all indicate that promoters of cancer signature genes, particularly those involved in the most aggressive types of tumors are enriched in NF-Y sites (Thomassen et al., 2008). Mirroring this trend, treating cells with anticancer drugs, or overexpressing growth suppressors, led to repression of NF-Y-dependent "cancer" genes (Blum et al., 2007; Yamanaka et al., 2009). Two facts are important: (i) the data, schematized in Figure 5, were collected by analyzing the promoters of overexpressed genes for a fixed set of TFBS, found in the TRANSFAC or JASPAR databases: the presence of E2F sites appears to be the only other common feature; (ii) *de novo* motif discovery, instead, identifies matrices in a unbiased way, without pre-set logos: the analysis of much larger – >6000 expression profiling – datasets of tumors also found NF-Y and E2F as the only enriched motifs (Rhodes et al., 2005; Sinha et al., 2008; Goodarzi et al., 2009). Therefore, the link between NF-Y and E2Fs mentioned above should be more closely detailed in the future, as it is an obvious nodal point of dysregulated expression in "cancer genes".

A specific example linking NF-Y to cancer progression came form studies on Menin, a tumor suppressor mutated in patients with endocrine cancers. Menin encodes a subunit of the MLL complex and overexpression represses transcription of the G2/M CyclinB2, whereas functional ablation of Menin increases it. Importantly, disease-related missense mutations abrogate this ability, possibly because DNA-binding of NF-Y, E2Fs and CREB is affected (Wu et al., 2010). Because NF-Y is able to recruit another MLL subunit, hASH2L (Fossati et al., 2011), it is tempting to hypothesize that Menin mutations affect the methyltransferase function of the MLL complex, its composition, or its ability to be recruited by NF-Y.

AEC. p63 is a master TF of all multilayered epithelia (Van Bockhoven et al., 2011). Dominant mutations in p63 cause a heterogeneous set of human ectodermal syndromes: specifically, mutations in the DBD *a-la* p53 cause EEC, in which limb abnormalities predominate, mutations in the C-terminal SAM domain cause AEC, in which skin is mostly affected. As a sequel to our findings of NF-Y/p53 interactions (Imbriano et al., 2005), we extended the observations to p63 and found that AEC, but not EEC missense p63 mutants are impaired in NF-Y binding *in vitro* and, *in vivo*, at least on G2/M promoters (Testoni & Mantovani, 2006). Mutagenesis identified the αC of NF-YC as important for the interaction, as for p53. Although overexpression of p63 AEC mutants alter transcription of CCAAT promoters in a NF-Y-dependent manner, it is unclear what might be the functional consequence of the heterozygous condition in skin cells, as found in patients.

Polyglutamine diseases

Huntington disease – HD – is an autosomal dominant neurodegenerative disorder associated with accumulation, due to expanded polyglutamine tracts, of mutant Huntingtin – HTT – in post-mitotic neurons of striatum and cortex. HTT was shown to aggregate with several TFs and cofactors with Gln-rich activation domains, resulting in impairment of their function (Dunah et al., 2002; Bradford et al., 2009). Initially, NF-Y was not among them (Chen-Plotkin et al., 2006), but a more recent, and thorough, study using mice models of HD, reported otherwise (Yamanaka et al., 2008). The presence of cytoplasmic aggregates containing the Gln-rich NF-YA and NF-YC subunits in neurons depleted the nuclear compartment, which led to an attenuation of CCAAT-binding activity. This is apparently perceived by Heat Shock genes – HSP40 and HSP70 – resulting in their decreased expression in neurons. These proteins are important chaperones that alleviate the consequences of malfolded protein aggregates, thus a negative loop would be established, enhancing cellular toxicity and, ultimately, the neurologic symptoms. The model is attractive, and is further supported by the finding that many genes whose expression is specifically altered in HD mice models do have classic CCAAT boxes. Another polyglutamine disease in which NF-Y was implicated is Spincerebellar Ataxia Type 17 (SCA17) caused by Gln expansion in the TBP gene. TBP is a TF that binds to TATA boxes, which we recently showed that for the most part, are present in CCAAT-less promoters (Dolfini et al., 2009). However,

TBP and NF-Y do collaborate in activation of a few genes, including those of the chaperones mentioned above (Landsberger & Wolffe, 1995), and indeed were shown to interact directly (Bellorini et al., 1997). Huang et al., recently reported *in vivo* experiments that are extremely similar to the ones described for HD, with Poly-Gln TBP causing NF-YA aggregates, and consequent degeneration of cerebellar Purkinje cells (Huang et al., 2011). Thus, it is important to define the specific contribution of NF-Y (as opposed to other Gln-rich TFs such as Sp1) to the pathogenesis of HD and SCA17.

A third polyglutamine disease in which malfunctioning of NF-Y was described is spinal and bulbar muscular atrophy – SBMA – in which motor neurons are affected (Katsuno et al., 2011). SBMA is caused by the expansion of CAG repeats in the androgen receptor – AR – gene, resulting in poly-Gln expansion. In this case, the pathogenetic mechanism apparently involves disregulation of the TGFβ pathway by the poly-Gln AR. The mutant AR represses TGFβ-IIR expression, affecting the whole pathway, by interfering with NF-YA/PCAF interactions (Katsuno et al., 2010). Interestingly, in a yeast model of poly-Gln proteins cytotoxicity is specifically suppressed by overexpression of HAP4, which improves cellular respiration by impinging on CCAAT promoters of cytochrome genes (Ocampo et al., 2010).

Leigh syndrome

Leigh syndrome is a devastating neurological genetic disease with an onset in early life, caused by a deficit in the function of mitochondria. The problems are due to the altered structure, or assembly, of complexes involved in oxidative phosphorylation, which are caused by mutations in many different genes. The single most frequent alteration is on Surf1, which codes for a protein presumably involved in the assembly of the complex: HAP4 was retrieved in genetic screens of complementation of a yeast strain mutated in the Surf1 homologue *Shy1* (Fontanesi et al., 2008). Reasoning that NF-YA was the activating subunit of the trimer in humans, fibroblasts derived from Surf1-deficient patients were transiently transfected and partial mitochondrial function reestablished. The underlying mechanism is not clear, but the likeliest hypothesis is that transcription of unknown genes that compensate for the Surf1 defect in the assembly of cytochrome complexes are improved by NF-YA overexpression.

Schizophrenia

Schizophrenia is a psychiatric disease of unknown origin, with multiple susceptibility genes shown to be associated. One of these genes is Dysbindin, a protein involved in neurotransmission; KO mice have schizophrenia-like symptoms. NF-YB was identified as a Dysbindin interactor in yeast 2-hybrids assays: Dysbindin and NF-Y regulate the CCAAT box of MARCKS, a gene whose expression is impaired in schizophrenic patients. The two proteins might also regulate additional genes involved in neuronal transmission altered in the disease (Okuda et al., 2010).

Diabetes

Two studies report circumstantial evidence linking altered NF-Y activity to diabetes. The expression of the CCAAT-dependent hepatic 3-hydroxy-3-methylglutaryl-CoA reductase – HMGR – gene is decreased in diabetic animals (Lagor et al., 2005). *In vivo* footprinting found SRE and NF-Y sites bound in normal, but not in diabetic animals, and insulin led to an increase of HMGR expression. Whether the lack of promoter binding by NF-Y and SREBP is the primary cause of the defect, or whether it is an epiphenomenon, is currently not clear. In another study, two single nucleotide polymorphisms – SNPs – of G6PC2, which encodes for an islet-specific glucose-6-phosphatase catalytic subunit, were found associated with elevated promoter activity and fasting plasma glucose (FPG): the mechanism is linked to increased NF-Y and Foxa2 DNA-binding (Bouatia-Naji et al., 2010).

Polymorphisms and mutations

Several additional reports indicate that the presence of specific SNPs in the NF-Y target sequence significantly affect the expression of genes involved in complex pathways. The rs2630578-C SNP in the BICD1 – Bicaudal-D homolog 1 – gene is linked to telomere shortening, a condition causing ageing: the variation potentially affects NF-Y-binding in an area positive for H3K4me3 (Mangino et al., 2008). Another example regards the cytochrome P450 2A6 – CYP2A6 – which encodes the nicotine C-oxidase, an important detoxifying enzyme, whose variable expression among individuals depends on polymorphisms in the promoter. The – 745A>G substitution disrupts NF-Y binding, affecting expression (von Richter et al., 2004). Additional examples of this sort fall in regulatory regions of the HaRAS and FerritinH genes (Faniello et al., 2002; Gorshkova et al., 2006). As genome sequencing of thousands of individuals progresses rapidly, we will probably witness a rapid accumulation of this type of association, with many other multi-genic diseases.

In the case of HPFH and β-thalassemia, mutations were reported a long time ago. Hereditary persistence of fetal hemoglobin – HPFH – is a condition in which the human CCAAT Aγ and Gγ genes, coding for fetal hemoglobin normally shut off at birth, continue production during adult life. Genetically, there are two forms: deletion-type, which is due to gross alterations in the δ and β gene region, and non-deletion types, which are caused by point mutations in the γ promoters (Forget, 1998). Among the latter, most are centered around the distal CCAAT box (Chassanidis et al., 2009). The model would be that mutations alter the binding of a repressive TF present in adult life, hence maintaining the gene expression. NF-Y is one of the TFs shown to be altered in binding to HPFH alleles (Liberati et al., 2001). As an activator, NF-Y would not be the primary culprit, but the recent data on the dual roles of NF-Y, as a positive and negative regulator, invite a reevaluation of this matter. The β-globin promoter also contains an important CCAAT box in a canonical – 80 region: a

mutation in this element was recently associated with β-thalassemia *intermedia*, a mild condition in which mRNA production is decreased, but not abolished (Chen et al., 2007).

In synthesis, alteration of NF-Y structure is not the direct cause of any specific disease, but evidence is accumulating that the trimer is involved in the development of several pathological conditions, whether because of altered DNA-binding in promoters, association with altered proteins, or altered gene expression patterns. This latter condition, relevant to tumor formation and/or progression, has lead to the concept that pharmacological interventions targeting NF-Y might be desirable in some conditions.

NF-Y and drugs

The importance of the CCAAT box in "cancer" genes spurred investigations aimed at developing drugs that alter DNA-binding patterns of NF-Y. Pyrrole-imidazole polyamides are sequence-specific compounds that bind DNA non covalently, affecting the DNA structure and, potentially, the activity of TFs (reviewed by Dervan & Edelson, 2003). In particular, polyamide compounds that specifically target the CCAAT box have been synthesized and tested (Buchmueller et al., 2005; Le et al., 2006; Hochhauser et al., 2007; Kotecha et al., 2008; Mackay et al., 2008). Some were shown to inhibit interactions with a broad range of CCAAT elements, *in vitro* and *in vivo* (Henry et al., 2004; Hochhauser et al., 2007); a second set was designed to target the crucial ICB2 CCAAT element of the Topo IIα promoter with high specificity *in vitro* (Franks et al., 2010). A completely different approach reached similar results, by using a miRNA – miR-485-3p – which targets NF-YB, and, therefore increases the levels of Topo IIα, making resistant cells susceptible to Topo IIα inhibitor drugs (Chen et al., 2011). Thus, the proof of principle of altering NF-Y binding *in vitro* and *in vivo* has been obtained, and this line of experiments shows promise to specifically target subclasses of CCAAT boxes, implicated in specific molecular pathways.

Taking a complementary approach, the laboratory of S. Schreiber has used yeast HAP3 to screen interacting compounds, from a library of >12,000, and identified Haptamide A. Subsequent chemical refinement improved affinity with a derivative – Haptamide B – which showed a KD in the high nanomolar range, and, most importantly, the capacity to affect *in vivo* function of a number of CCAAT-dependent promoters in yeast (Koehler et al., 2003). NF-YB was also identified in screenings of proteins interacting with HMN176 (Tanaka et al., 1999), the active metabolite of the antitumor compound HNM-214, currently developed as a Polo-like Kinase (PLK) inhibitor (Schöffski, 2009). HNM-176 inhibits NF-Y binding *in vitro* and MDR1 overexpression in cancer cell lines, restoring chemosensitivity to other anti-cancer agents (Tanaka et al., 2003).

Another set of compounds whose activity clearly alters NF-Y activity are HDAC-inhibitors (HDAC-I). In most studies, the Class I and II inhibitor TSA was used, while SAHA and VPA (current pharmaceutical compounds) have also been employed. Inhibition of HDACs leads to increase in promoter activity of a plethora of genes including: MDR1, TGFβ–RII, RhoB, GADD45, mRR2, PDK4, TSP1, FPGS, TBP2, Gdf11, hPTTG, FerritinH, HSP70, RGS4 (Jin and Scotto, 1998; Park et al., 2002; Butler et al., 2002; Hirose et al., 2003; Wang et al., 2003; Zhang et al., 2004; Liu et al., 2004; Huang et al., 2005; Tabe et al., 2006; Kwon et al., 2006; Kang et al., 2008; Li et al., 2009; Leclerc et al., 2010; Wang et al., 2010; Marinova et al., 2011; Yang et al., 2010). Whenever sought, the activating HDAC-I function was mapped to CCAAT boxes and NF-Y, often in combination with other TFs such as Sp1 (Huang et al., 2005) or Oct (Hirose et al., 2003; Campanero et al., 2008). The combination of these elements were responsible for the recruitment of KATs, in most cases KAT3B (p300). The presumed *scenario* is that HDAC-I non-specifically increases histone H3 and H4 acetylation and this leads to "opening" of chromatin, and stronger function of the TFs. Interestingly, recent reports indicate that local levels of histone acetylation are invariant, whereas the recruitment of NF-YA is robustly increased (Wang et al., 2010; Yang et al., 2010); in the case of RSG4, this is accompanied by increased NF-YA acetylation. Furthermore, interactions between NF-Y and HDACs and KATs are regulated by HDAC-I (Peng et al., 2007): bearing in mind that NF-YB and NF-YA are acetylated (Li et al., 1998; Manni et al., 2008), it seems reasonable to imagine that HDAC inhibition could impact on acetylation of NF-Y, as well as of core histones. This topic is of great importance, since it might be desirable to select compounds that affect the trimer more specifically.

Various other compounds have been shown to alter the activity of NF-Y. Genistein represses ER-stress genes (Zhou & Lee, 1998), but, similarly to daidzein and flavone, it activates, BSP expression through NF-Y (Shimizu & Ogata, 2002; Sharina et al., 2003). Quercetin represses NF-Y binding to Cyclin B2, without changing the overall levels of the trimer (Jeong et al., 2009; Kim et al., 2007). Atorvastatin has no consequences on NF-Y (Rodrigues et al., 2009), but Simvastatin, A23183, Okadaic Acid, TNFα and Nitric oxide treatments lead to a poorly understood CCAAT-mediated repression of various promoters (Zhu et al., 2007a, 2007b; Finch et al., 2001; Morin et al., 1995; Park et al., 2002; Park et al., 2011; Harari & Liao 2004). ET743 (Trabectedin) affects induction of the CCAAT containing HSP70 and MDR1 promoters (Minuzzo et al., 2000; Jin et al., 2000), but the inhibition of activated transcription is also observed in CCAAT-less units as well (Friedman et al., 2002; Minuzzo et al., 2005). The mechanisms behind the above observations, in particular whether the effects are exerted through direct binding to any of the subunits, is completely unknown. Altogether, the numerous data on the effects of compounds and drugs on NF-Y activity await a rationalization

by *in silico* experiments with available structures of NF-Y, and *in vitro* biochemical assays. These types of predictive assays could also lead to the identification and testing of new compounds targeting the subunits in more informed ways.

Future directions

While a considerable amount of work has placed NF-Y at the center of transcriptional activation of some 30% of genes in eukaryotes, a definition of the fine mechanisms will require: (i) a structural understanding on how NF-YA binds to the HFD dimer, how the trimer contacts DNA, and what are the relationships with the other core and variant histones. (ii) A knowledge of the various PTMs of the subunits, their "writers" and "readers", and their specific function. (iii) The dissection of the *in vivo* interplay with hundreds of TFs, cofactors and epigenetic marks, uncovered by ChIP-Seq studies. (iv) Studies on the role of miRNAs on NF-Y biology, an area that has received modest attention so far. These advances should improve our knowledge of the pathogenesis of the many diseases in which NF-Y is involved, while at the same time lead to the possibility of designing compounds to alter, positively or negatively, NF-Y activity in such pathological conditions.

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